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Both the Suprachiasmatic Nucleus and the Superior Ovarian Nerve Contribute to the Processes of Ovulation and Steroid Hormone Secretion on Proestrus

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Abstract

The aims of the present study were to analyze if the superior ovarian nerve (SON) plays a role in the neural signals from suprachiasmatic nucleus (SCN) that lead to ovulation and ovarian steroids secretion on proestrus day. Rats on proestrus day were treated at 11.00 to 11.30 or 17.00 to 17.30 hours with 1 of the 3 experimental procedures (1) unilateral or bilateral SON sectioning, (2) unilateral or bilateral injury to the SCN, or (3) unilateral injury to the SCN followed by unilateral sectioning of the SON ipsilateral to the treated SCN. Treatments were evaluated 24 hours after surgical procedures. Compared to laparotomized animals, right or bilateral SON sectioning treatment at 17.00 hours resulted in lower ovulation rates and number of ova shed by the right ovary. The ovaries of nonovulating animals showed early follicular luteinization signs and trapped ova. Bilateral SCN injury treatment at 11.00 hours resulted in anovulation; whereas right SCN injury treatment, with or without right SON sectioning, resulted in a lower number of ova shed. Injecting luteinizing hormone-releasing hormone to animals with bilateral SCN injury restored ovulation. In rats with unilateral or bilateral SON sectioning, or with injury to the SCN with or without unilateral sectioning of the SON, the effects on hormone levels depended of the hormone studied and the time of day treatment was performed. The present results suggest that on proestrus day, the role of the right or both SON in ovulation and steroid hormone secretion regulation takes place through different neuroendocrine mechanisms from SCN.

Keywords

superior ovarian nerve, suprachiasmatic nucleus, ovulation, steroidogenesis, proestrus

Introduction

Ovulation and steroids hormones secretion are regulated by hormonal and neural signals that mainly arise from the hypothalamus, pituitary, ovary, and adrenals.[1,2] Neural signals arrive to the ovaries through the superior ovarian nerve (SON), the ovarian plexus nerve (OPN), and the vagus nerve.[3] The SON and OPN have their neurons located mainly in the celiac-superior mesenteric ganglia.[3-5]

The SON provides the ovary with fibers containing catecholamines, vasoactive intestinal peptide (VIP), and neuropeptide Y (NPY).[5] The fibers of the SON are mainly distributed in the perifollicular theca layer, in close relation with the theca internal cells, while the noradrenergic fibers penetrating the ovary through the OPN are mainly perivascular.[3,6]

We have previously shown that 24 and 72 hours after unilateral or bilateral sectioning of the SON noradrenaline (NA) levels in the denervated ovary were lower than in untouched (control) and laparotomized rats.[7] According to Aguado and Ojeda,[8] adult rats on proestrus day with bilateral SON sectioning treatment at 11.00 hours showed lower progesterone and estradiol levels 4 minutes after treatment, whereas the same treatment performed at 16.00 hours resulted in a brief (8 minutes) progesterone secretion decrease and induced a prolonged estradiol decline. In rats on estrus day, the bilateral SON sectioning treatment at 11.00 hours did not alter progesterone or estradiol levels in serum.[8]

According to Flores et al,[9] compared to their corresponding laparotomized treatment group, unilateral SON sectioning

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treatment to adult rats on proestrus at 13.00 to 13.15 hours had different effects on testosterone levels depending on the SON sectioned. One hour after right SON sectioning treatment, testosterone levels were lower while left SON sectioning did not modify it. No changes in estradiol levels were observed.

Kagitani et al[10] reported that in rats on estrous day right SON electrical stimulation reduced estradiol secretion rates of from the right ovary, whereas Uchida and Kagitani[11] showed that the right SON has an inhibitory role in ovarian testosterone secretion.

Taken together, the evidence suggests that a circadian mechanism may regulate ovarian functions and that such regulation can occur in any component of the hypothalamic–pituitary–ovarian axis, as proposed by Kennaway[12] and Sellix.[13]

The participation of the hypothalamus in the regulation of gonadotropin secretion and ovulation includes the preoptic-anterior hypothalamic area,[14] the arcuate nucleus,[15] the anteroventral periventricular nucleus,[16] and the suprachiasmatic nucleus (SCN).

In mammals, a cell-intrinsic molecular circadian pacemaker located in the SCN is part of the mechanism that allows them to anticipate changes in the environment and to regulate female reproductive functions.[2] In rats, the chronic bilateral lesion of the SCN results in persistent vaginal estrus,[17] anovulation,[18,19] and blockage of the gonadotropin-releasing hormone (GnRH) surge; this interrupts the secretion timing of the luteinizing hormone (LH).[20,21]

Terasawa et al[22] showed that the acute bilateral electrolytic lesion to the SCN on the morning of proestrus (09.00-10.00 hours) failed to block ovulation on the morning of estrus day. These results contradict the findings of Kimura and Kawakami,[23] who reported that in rats on proestrus the bilateral lesion to the SCN at 12.00 to 14.00 hours produced an acute blocked of the preovulatory LH surge and blocked ovulation.

According to Gerendai et al,[24] there is neuromorphological evidence of the transneuronal innervation asymmetry of the ovary and on the asymmetry of the hypothalamus and extrahypothalamic structures transneuronally connected with the ovary. Furthermore, data have been published on the side-linked regulation asymmetry of the circadian rhythm in the SCN.[25-27]

The SCN is connected to many peripheral tissues via the sympathetic and parasympathetic branches of the autonomic nervous system.[24,28-30] To our knowledge, there are no anatomical evidences showing the neural connections between the SCN and the ovary. Kennaway[12] proposed that it is reasonable to expect that the sympathetic innervation of the ovary and uterus may also be influenced by the SCN. Sellix[13] suggested that the SCN may modulate the timing of the ovarian clock via autonomic nervous cues (sympathetic and parasympathetic nervous system).

Based on the proposals of Kennaway[12] and Sellix[13], the aims of the present study were to analyze the influences of the SCN on the SON's sympathetic innervation of the ovary in regulating ovarian functions in rats on proestrus day before (at 11.00 hours) or after (at 17.00 hours) the preovulatory LH peak.

To this end, 3 experimental procedures to evaluate their effects on ovulation and steroid hormone secretion were conducted in rats on proestrus day. The 3 experiments consisted of (1) unilateral or bilateral sectioning to the SON at 11.00 or 17.00 hours, (2) unilateral or bilateral injury to the SCN at 11.00 or 17.00 hours, and (3) unilateral injury to the SCN followed by sectioning the SON ipsilateral to the injured SCN.

Materials and Methods

All experiments were carried out in strict accordance with the Mexican Law of Animal Treatment and Protection Guidelines and followed the Mexican Official Standard NOM-062-ZOO-1999 specifications. The Institutional Committee for the Care and Use of Animals of the Facultad de Estudios Superiores Zaragoza approved all experimental protocols. All possible efforts were made to minimize the number of animals used and their suffering.

This study was performed with adult, female, CIIZ-V strain rats weighing 230 to 260 g from our own breeding stock. The animals were maintained under controlled light (on from 05.00 to 19.00 hours) and temperature (22°C $\pm$ 2°C) conditions, with free access to food (Purina S.A., Mexico) and tap water. The animals' estrous cycles were monitored by cytological examination of daily vaginal smears. Only rats showing at least 2 consecutive 4-day estrus cycles were used in the experiment. The study was made with rats on proestrus day. The rats were anaesthetized with ether and surgical procedures were performed between 11.00 and 11.30 or 17.00 and 17.30 hours. Animals were sacrificed 24 hours after surgery treatment. The experimental protocol (Figure 1) in this article indicates the number of animals used in each experimental group.

Groups of animals were randomly allotted to each of the following experimental groups.

Unilateral or Bilateral SON Sectioning

Rats were allotted to 1 of the 6 surgery treatment groups—left, right, or bilateral SON sectioning or laparotomy (Figure 1A). Following previously described methodologies,[31] a unilateral dorsolateral incision was performed 2 cm below the last rib on the left, right, or bilateral abdominal wall. The incision affected skin, muscle, and peritoneum. The left, right, or both ovaries were exposed and with the aid of fine forceps the ovarian ligament was sectioned approximately 1 cm from the ovary. The gonad was immediately returned to the abdominal cavity and the wound was sealed. The sham section of the SON treatment consisted of performing a dorsolateral incision on the left, right, or bilateral abdominal wall (laparotomy). No organs were manipulated. The wound was subsequently sealed.

Unilateral or Bilateral Injury to the SCN

Animals were allotted to 1 of the 6 surgery treatment groups—left, right, or bilateral SCN injury or sham (Figure 1B). The animals were anaesthetized and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, California). The skin of the skull was sectioned and the left and/or right side of the skull

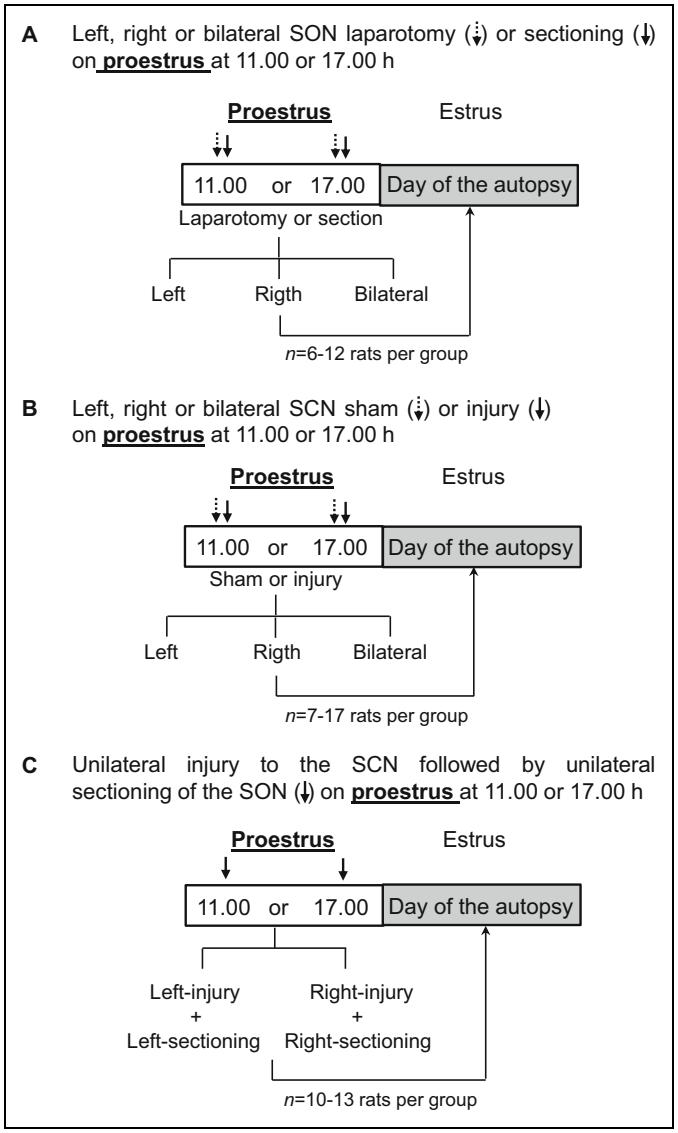

Figure 1. Schematic representation of the treatment design. A total of 28 groups of rats (n = 300) were randomly allotted to each of the following experimental procedures: (A) laparotomy or sectioning of the left, right, or bilateral superior ovarian nerve (SON) at 11.00 or 17.00 hours; (B) sham or injury treatment to the left, right, or bilateral suprachiasmatic nucleus (SCN); (C) unilateral injury to the SCN followed by unilaterally sectioning of the SON ipsilateral to the injured nucleus. All animals were sacrificed on the predicted estrus day (24 hours after surgery).

was drilled with a 1-mm bit. Subsequently, a thermic electrode insulated except at the 0.2-mm tip (thermocouple zone [TCZ], tip diameter 0.3 mm; Radionics, Inc, Burlington, Massachusetts) was connected to a Model RFG 4A Research Radio Frequency Lesion Generator System (Radionics, Inc) and lowered into the left, right, or both sides of the SCN. The SCN was located according to the coordinates of the Paxinos and Watson atlas[32] (left SCN: −0.3 mm anterior–posterior to the bregma, +0.3 mm lateral–medial to the bregma, and −9.1 mm below to the bregma; right SCN: −0.3 mm anterior–posterior to the bregma, −0.4 mm lateral–medial, and −9.1 mm below to the bregma). The injury into the left, right, or both sides of the SCN was produced by passing current of 10 mA at a voltage of about 20 V that heated the electrode tip to a constant temperature of 90°C for 15 seconds. After surgery, the electrode was removed and the wound sutured.

The sham surgery into the SCN consisted of the same procedures described earlier, except that no current was passed through the electrode.

Luteinizing Hormone–Releasing Hormone Treatment to Nonovulating SCN-Treated Animals

The SCN injuries that blocked ovulation were identified and the treatments were replicated in a group of 6 animals. At 14.00 hours, approximately 3 hours after SCN injury treatment, the rats were subcutaneously injected with 3.7 μg/kg body weight of synthetic LH–RH (Sigma Chemical Co, St Louis, Missouri). These animals were sacrificed the next morning after treatment.

Unilateral Injury to the SCN Followed by Unilaterally Sectioning of the SON Ipsilateral to the Injured SCN (SCN Injury + SON Sectioning)

Following the procedures described earlier, groups of animals were treated with a unilateral injury to the SCN followed by sectioning of the SON ipsilateral to the injured SCN (*left*: L-SCN injury + L-SON sectioning; *right*: R-SCN injury + R-SON sectioning). Procedures were performed between 11.00 and 11.30 or 17.00 and 17.30 hours (Figure 1C).

Autopsy Procedures

Groups of animals from each experimental group were sacrificed by decapitation 24 hours after the surgery. The blood of the trunk from each animal was collected and centrifuged at 3500 rpm for 15 minutes. The serum was stored at −20°C until the progesterone, testosterone, and estradiol levels were measured by radioimmunoassay. During autopsy the effectiveness of the SON sectioning procedure was confirmed by verifying the free movement of the ovary in the abdominal cavity. The oviducts were dissected and the number of ova present was counted with the aid of a stereoscopic microscope (Nikon, Model C-PS, Tokyo, Japan). Ovarian morphology was assessed in nonovulating animals.

Ovarian Morphology Assessment

The ovaries from each nonovulating experimental rats were removed and cleaned of adherent fat tissue, subsequently immersed in Bouin's fixative solution for 24 hours, dehydrated and embedded in paraffin. Ten microns-thick serial histological sections were made and stained with hematoxylin–eosin. With the aid of a Nikon binocular microscope, all histological sections were analyzed for the presence of new corpora lutea and follicles with enclosed oocyte.

Confirming the Location of the SCN Injury

The accuracy of the SCN injury and sham surgery treatments was verified by removing the brains of treated rats, fixing them in 10% formaldehyde, and subsequently obtaining 100-μm thick slices using a vibratome (Series 3000; Technical Products International, Inc, St Louis, Missouri). Brain slices were stained with 1% cresyl violet. The location of the injured zone or the trajectory of the electrode (sham surgery) was assessed histologically with the aid of a stereoscopic microscope. The SCN measures between 500 and 600 μm in our strain of rats.

Images documenting the injured zone or the electrode path were obtained with a digital camera (HP Photosmart M637). Figure 2 shows the electrode path and the injured area in the SCN. Only those animals with complete SCN lesions were included in the analysis.

Hormone Concentration Measurements

The serum levels of progesterone (ng/mL), testosterone, and estradiol (pg/mL) were measured using solid-phase radioimmunoassay with kits purchased from Diagnostic Products (Los Angeles, California). The intra- and interassay coefficients of variation were 6.58% and 7.42% for progesterone; 7.85% and 8.76% for testosterone; and 7.54% and 8.21% for estradiol.

Statistical Analysis

The statistical analyses were performed using the GraphPad Prism 6 Software. The ovulation rates (the number of ovulating animals/the number of treated animals) were analyzed using Fisher exact probability or χ^2 test. Data on the number of ova shed were analyzed using Kruskal-Wallis test followed by Mann-Whitney U test. Data on progesterone, testosterone, and estradiol serum levels were analyzed with 1-way analysis of variance (ANOVA) followed by Tukey test when assessing the effects of treatments, and a 2-way ANOVA test was used when assessing the effects of the same treatments at different hours of the day (11.00 vs 17.00 hours). A P value less than .05 was considered statistically significant.

Results

Effects of Unilateral or Bilateral SON Sectioning

Ovulation. Compared to the corresponding left laparotomy group, left SON sectioning at 11.00 or 17.00 hours did not modify ovulation rate nor the number of ova shed. Ovulation rate by rats with right SON sectioning at 11.00 hours was similar to the laparotomized group, though the number of ova shed by the right ovary was lower. The number of ova shed by the left ovary in rats with bilateral SON sectioning was lower than in the bilateral laparotomized group (Table 1).

Compared to their respective laparotomized group, rats with right or bilateral SON sectioning treatment at 17.00 hours resulted in lower ovulation rate and number of ova shed by the right ovary (Table 1).

Ovarian morphology. Figure 3 shows luteinized preovulatory follicles with trapped ova in nonovulating rats with unilateral or bilateral SON sectioning treatment at 11.00 hours (Figure 3A). No newly formed corpora lutea were observed. In rats treated at 17.00 hours (Figure 3B), the largest ovaries showed signs of early luteinization and trapped ova.

Effects of SON sectioning on hormone levels

SON sectioning at 11.00 hours. Rats treated with unilateral (left or right) or bilateral SON sectioning showed similar progesterone and testosterone levels as its corresponding laparotomized group. Rats treated with right SON sectioning treatment showed lower estradiol levels than its corresponding laparotomized group (Figure 4).

SON sectioning at 17.00 hours. Rats treated with unilateral (left or right) or bilateral SON sectioning showed similar progesterone levels as its corresponding laparotomized group. Bilateral SON sectioning treatment resulted in higher testosterone levels than in bilaterally laparotomized animals. Compared to the corresponding laparotomized treatment group, estradiol levels were lower in animals with left SON sectioning treatment and higher in rats with bilateral SON sectioning treatment (Figure 4).

The 2-way ANOVA test showed that the animals with bilateral SON sectioning treatment at 11.00 hours yielded lower testosterone levels than animals treated at 17.00 hours ($F_{1,\ 24} = 31.37$; $P < .0001$) with significant effects between treatments and hours ($F_{2,\ 48} = 6.813$; $P < .0045$). Animals with right SON sectioning at 11.00 hours showed lower estradiol levels than rats treated at 17.00 hours ($F_{1,\ 31} = 9.010$; $P < .0053$) but no apparent effects were observed between treatments and hours ($F_{2,\ 31} = 1.433$; $P < .2539$).

Effects of Unilateral or Bilateral SCN Injury

Ovulation. No difference in ovulation rate or number of ova shed was observed between left sham surgery and left SCN injured animals treated at 11.00 or 17.00 hours.

Compared to right sham surgery rats, animals with right SCN injury treatment at 11.00 or 17.00 hours showed similar ovulation rate but lower number of ova shed (Table 2).

Bilateral SCN injury treatment at 11.00 hours resulted in anovulation (0 of 6 vs 10 of 10). Injecting synthetic LH–RH to rats with bilateral SCN injury at 11.00 hours restored ovulation (5 of 5 vs 0 of 6; $P < .005$, Fisher exact probability test), and the total number of ova shed was similar to the bilateral sham surgery treatment group (10.5 ± 0.9 vs 10.6 ± 0.7).

SCN injury effects on hormone levels

SCN injury at 11.00 hours. Rats with left SCN injury treatment showed lower progesterone levels than its corresponding sham surgery group. Such difference was not observed in rats with right or bilateral SCN injury treatment.

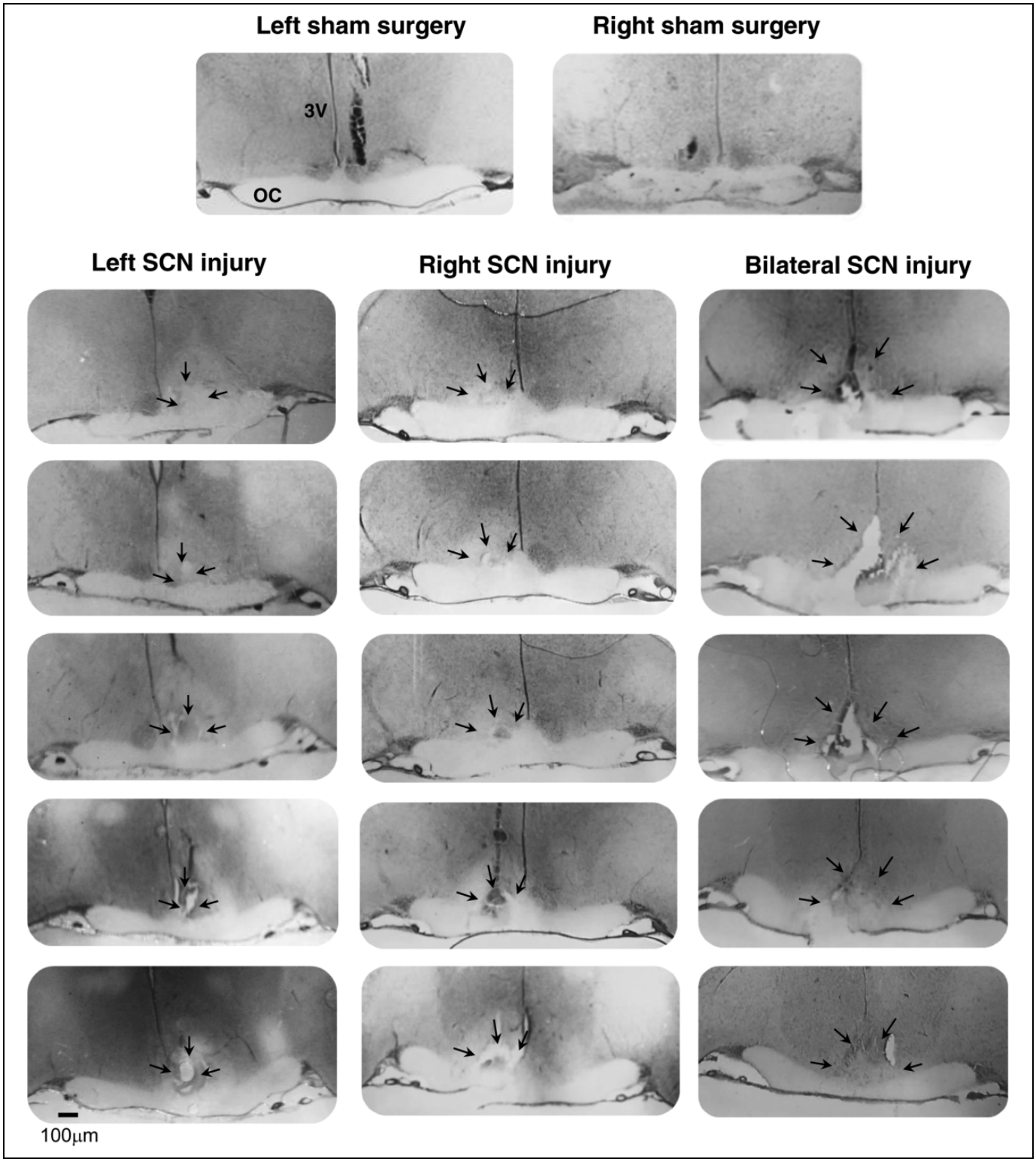

Figure 2. Image of brain coronal sections showing the trajectory of the electrode (sham surgery) and the injured zone. Coronal sections showing the rat's suprachiasmatic nucleus (SCN) stained with cresyl violet, and the electrode trajectory (sham surgery) to the SCN or the right, left, or bilateral SCN injury zone (arrows). OC indicates optic chiasma; 3V, third ventricle. 4× microscopic lens, scale bar = 100 μm.

Compared to its corresponding sham surgery group, testosterone levels were lower in animals with right SCN injury treatment and higher in the bilateral SCN injury group.

Rats with a bilateral SCN injury treatment showed higher estradiol levels than the bilateral sham surgery group (Figure 5).

Table 1. Ovulatory Response of the Animals With Unilateral or Bilateral Sectioning of the Superior Ovarian Nerve (SON).[a]

	11.00 hours				17.00 hours			
	Ovulation Rate per Ovary		Number of Ova Shed per Ovary		Ovulation Rate per Ovary		Number of Ova Shed per Ovary	
Groups	Left	Right	Left	Right	Left	Right	Left	Right
L-LAP	9/10	10/10	6.1 ± 0.5	6.5 ± 0.5	9/10	9/10	6.0 ± 0.6	5.4 ± 0.7
L-sectioning	9/14	13/14	4.8 ± 0.6	6.8 ± 0.7	12/17	11/17	4.4 ± 0.7	5.5 ± 0.7
R-LAP	10/10	9/10	5.6 ± 0.7	7.0 ± 0.6	7/10	9/10	5.7 ± 0.5	6.6 ± 0.8
R-sectioning	14/16	8/16	4.1 ± 0.6	4.4 ± 0.7[b]	11/15	7/15[c]	5.6 ± 0.6	3.6 ± 0.9[b]
B-LAP	10/10	9/9	6.7 ± 0.7	5.6 ± 1.0	7/7	7/7	4.6 ± 0.8	6.0 ± 1.1
B-sectioning	9/10	9/10	4.7 ± 0.5[b]	4.2 ± 0.2	10/14	6/14[c]	4.8 ± 0.5	2.3 ± 0.3[b]

[a]Ovulation rates and numbers of ova shed (means ± standard error of the means) by the left or right ovary of rats with left (L-LAP), right (R-LAP), or bilateral (B-LAP) laparotomy or with left (L-sectioning), right (R-sectioning), or bilateral (B-sectioning) SON sectioning treatment. Treatments were performed to rats on proestrus day between 11.00 and 11.30 or 17.00 and 17.30 hours. The animals were sacrificed 24 hours after surgery.
[b]$P < .05$ versus corresponding laparotomized group (Kruskal-Wallis test followed by Mann-Whitney *U* test).
[c]$P = .0098$ versus corresponding laparotomized group (χ^2 test).

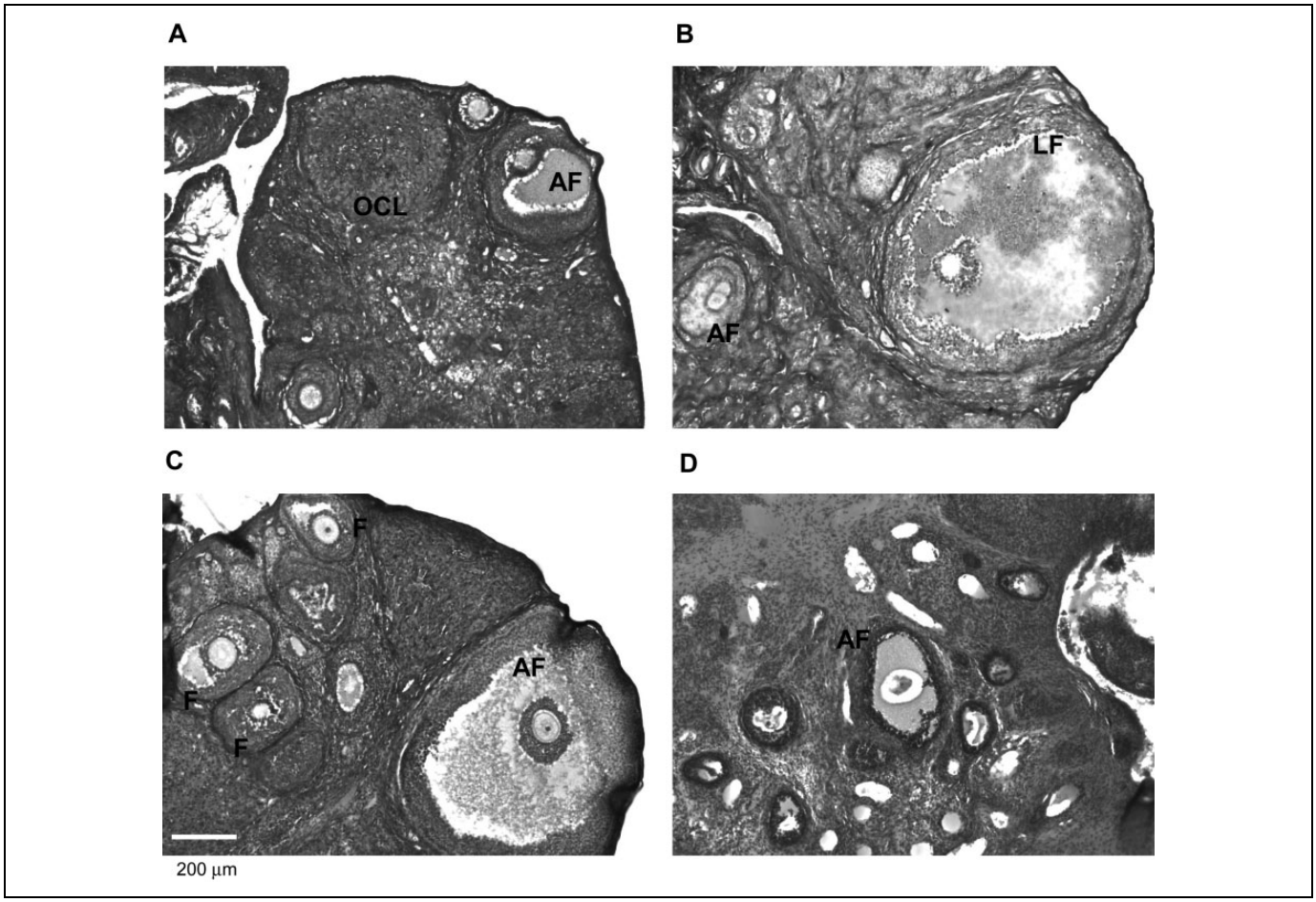

Figure 3. Ovarian histology of rats treated with right or bilateral superior ovarian nerve (SON) sectioning or treated with right suprachiasmatic nucleus (SCN) injury followed by right SON sectioning. Micrographs correspond to sections of the ovary, of 10 μm thick, stained by hematoxylin–eosin. Ovary from a right or bilateral SON sectioning treated rat at 11.00 hours (A) or 17.00 hours (B). Ovary from a rat treated with right SCN injury followed by right SON sectioning at 11.00 hours (C) or 17.00 hours (D). Treatments were performed to rats on proestrus day between 11.00 and 11.30 or 17.00 and 17.30 hours. The animals were sacrificed 24 hours after surgery. AF indicates atretic follicle; F, normal follicle; LF, luteinized follicle; OCL, old corpora lutea. 10× microscopic lens, scale bar = 200 μm.

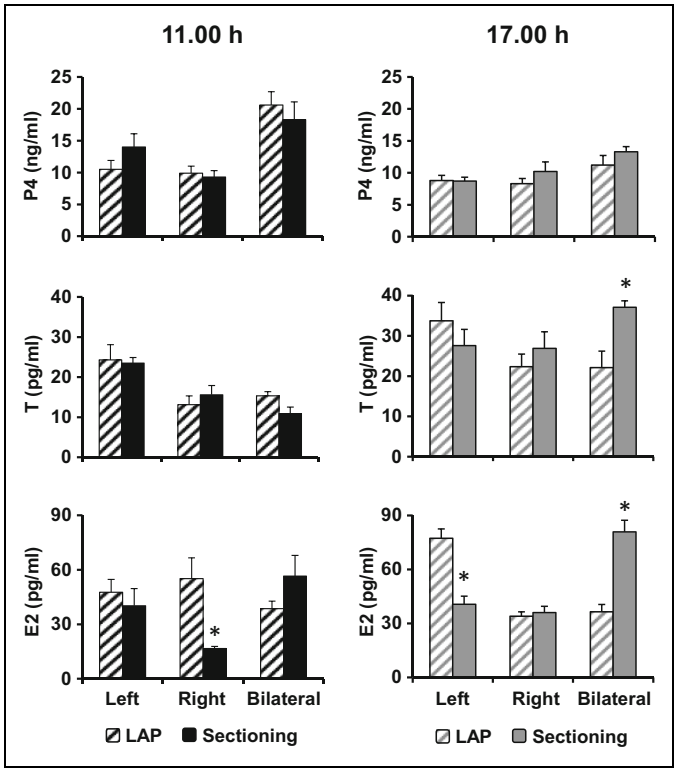

Figure 4. Effects of unilateral (left or right) or bilateral superior ovarian nerve (SON) sectioning treatment on hormones levels. Mean ± standard error of the mean of progesterone (P4, ng/mL), testosterone (T, pg/mL), and estradiol (E2, pg/mL) serum levels in rats with left, right, or bilateral laparotomy (LAP) or with left, right, or bilateral SON sectioning. Treatments were performed with rats on proestrus between 11.00 and 11.30 or 17.00 and 17.30 hours. The animals were sacrificed 24 hours after treatment (n = 8 per group). *$P < .05$ versus corresponding laparotomized group (1-way analysis of variance followed by Tukey test).

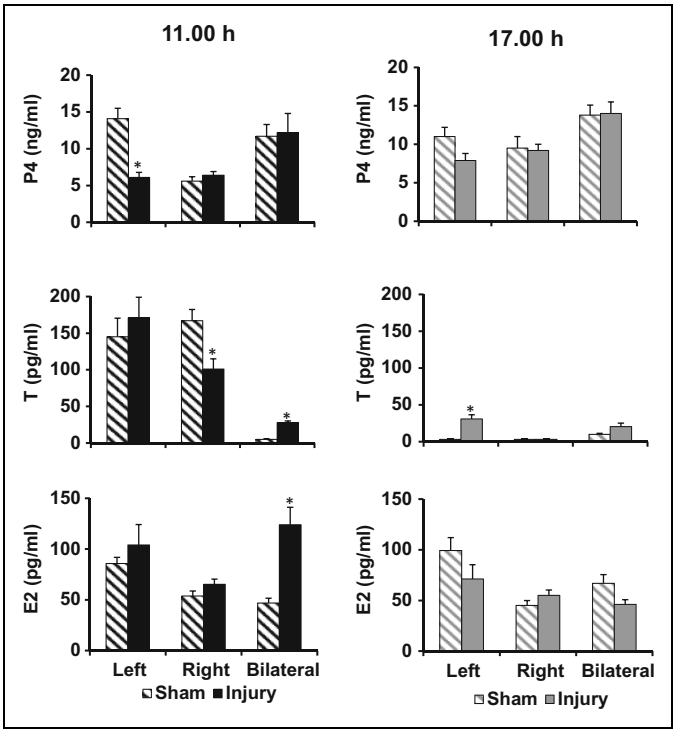

Figure 5. Unilateral or bilateral suprachiasmatic nucleus (SCN) injury treatment effects on hormones levels. Mean ± standard error of the mean of progesterone (P4, ng/mL), testosterone (T, pg/mL), and estradiol (E2, pg/mL) serum levels in rats with left, right, or bilateral sham surgery treatment or with left, right, or bilateral SCN injury treatment. Treatments were performed to rats on proestrus day between 11.00 and 11.30 or 17.00 and 17.30 hours. The animals were sacrificed 24 hours after treatment (n = 8 per group). *$P < .05$ versus corresponding sham group (1-way analysis of variance test followed by Tukey test).

Table 2. Ovulatory Response of the Animals With Unilateral or Bilateral Injury to the Suprachiasmatic Nucleus (SCN).[a]

	11.00 hours			17.00 hours		
		Number of Ova Shed per Ovary			Number of Ova Shed per Ovary	
Groups	Ovulation Rate	Left	Right	Ovulation Rate	Left	Right
L-sham	12/12	5.5 ± 0.6	5.8 ± 0.8	9/9	5.1 ± 0.7	5.4 ± 0.7
L-injury	8/10	6.0 ± 0.7	4.6 ± 0.3	9/9	6.2 ± 0.5	6.3 ± 0.8
R-sham	8/8	5.7 ± 0.5	5.1 ± 0.4	11/11	6.5 ± 0.4	6.2 ± 0.6
R-injury	8/11	5.1 ± 0.5	3.5 ± 0.5[b]	11/11	4.5 ± 0.6[b]	5.2 ± 0.8
B-sham	10/10	6.0 ± 0.5	5.7 ± 0.5	11/11	4.5 ± 0.6	5.7 ± 0.8
B-injury	0/6[c]	0	0	11/11	5.8 ± 0.6	6.1 ± 0.6

[a]Ovulation rates and numbers of ova shed (means ± standard error of the means) by the left or right ovary of rats with left (L-sham), right (R-sham), or bilateral (B-sham) sham surgery or with left (L-injury), right (R-injury), or bilateral (B-injury) injury to the SCN. Treatments were performed to rats on proestrus day between 11.00 and 11.30 or 17.00 and 17.30 hours. The animals were sacrificed 24 hours after surgery.
[b]$P < .05$ versus corresponding sham group (Kruskal-Wallis test followed by Mann-Whitney *U* test).
[c]$P = .0098$ versus corresponding sham group (χ^2 test).

Table 3. Ovulatory Response of the Animals With Unilateral Sectioning of the Superior Ovarian Nerve (SON) Ipsilateral to the Injured Suprachiasmatic Nucleus (SCN).[a]

	11.00 hours			17.00 hours		
		Number of Ova Shed per Ovary			Number of Ova Shed per Ovary	
Groups	Ovulation Rate	Left	Right	Ovulation Rate	Left	Right
L-sectioning	13/14	4.8 ± 0.6	6.8 ± 0.7	12/17	4.4 ± 0.7	5.5 ± 0.7
L-injury	8/10	6.0 ± 0.7	4.6 ± 0.3	9/9	6.2 ± 0.5	6.3 ± 0.8
L-injury + L-sectioning	7/11	4.0 ± 0.9	5.0 ± 1.3	12/13	5.4 ± 0.8	6.9 ± 1.0
R-sectioning	14/16	4.1 ± 0.6	4.4 ± 0.7	11/15	5.6 ± 0.6	3.6 ± 0.9
R-injury	8/11	5.1 ± 0.5	3.5 ± 0.5	11/11	4.5 ± 0.6	5.2 ± 0.8
R-injury + R-sectioning	3/10[b]	5.5 ± 0.4	1.7 ± 0.3[c]	10/11	6.9 ± 0.9	4.6 ± 0.9

[a]Ovulation rates and numbers of ova shed (means ± standard error of the means) by the left or right ovary of rats treated with left (L-sectioning) or right (R-sectioning) SON sectioning; with left (L-injury) or right (R-injury) SCN injury or with unilateral SCN injury followed by sectioning of the SON ipsilateral to the injured SCN (L-injury + L-sectioning; R-injury + R-sectioning). Treatments were performed to rats on proestrus day between 11.00 and 11.30 or 17.00 and 17.30 hours. The animals were sacrificed 24 hours after surgery.
[b]$P < .05$ versus R-Sectioning (Fisher exact test).
[c]$P < .05$ versus right ovary of animals with R-Sectioning or R-Injury (Kruskal-Wallis test followed by Mann-Whitney *U* test).

SCN injury at 17.00 hours. Rats in the unilateral and bilateral SCN injury treatment groups showed similar progesterone and estradiol levels as its corresponding sham surgery group. Rats with left SCN injury treatment showed higher testosterone levels than its corresponding sham surgery group. Such difference was not observed in rats with right or bilateral SCN injury treatment (Figure 5).

The 2-way ANOVA test shows that unilateral SCN injury treatment at 11.00 hours resulted in higher testosterone levels than in animals treated at 17.00 hours ($F_{1, 25} = 44.46$; $P < .0001$) but no apparent effects were observed between treatments and hours ($F_{1, 25} = 1.187$, $P < .2863$). Animals with bilateral SCN injury treatment at 11.00 hours showed higher estradiol levels than rats treated at 17.00 hours ($F_{1, 31} = 13.85$, $P < .0008$) and significant effects between treatments and hours ($F_{2, 31} = 3.490$; $P < .0429$).

Effects of Unilateral SON Sectioning to Animals With Unilateral SCN Injury Treatment

Ovulation. Similar ovulation rate and number of ova shed were observed between rats treated with left SCN injury, left SON sectioning, or left SCN injury followed by left SON sectioning at 11.00 or 17.00 hours (Table 3). Rats with right SCN injury followed by right SON sectioning treatment at 11.00 hours showed a lower ovulation rate than the animals with only right SON sectioning, whereas the number of ova shed was lower than in rats treated with only right SON sectioning or right SCN injury (Table 3).

Ovarian morphology. Figure 3 shows the large preovulatory follicles in nonovulating rats treated at 11.00 hours with right SCN injury followed by right SON sectioning (Figure 3C) showed signs of atresia and free ova in the follicular antrum, while atretic follicles were observed in animals treated at 17.00 hours (Figure 3D).

Effects of the SON sectioning to animals with SCN injury on hormone levels

SCN injury + SON sectioning treatment at 11.00 hours. Progesterone levels were higher in rats treated with left SCN injury followed by left SON sectioning than in rats with only left SCN injury treatment. Progesterone levels were lower in rats treated with right SCN injury followed by right SON sectioning than in rats with left SCN injury followed by left SON sectioning treatment. Regardless of the SCN side treated, testosterone and estradiol levels were lower in rats treated with unilateral SCN injury followed by unilateral SON sectioning than rats with unilateral SCN injury treatment only (Figure 6).

SCN injury + SON sectioning treatment at 17.00 hours. No difference in progesterone levels was observed between animals with unilateral SCN injury followed by unilateral SON sectioning treated and rats with unilateral SCN injury or unilateral SON sectioning.

Testosterone levels in rats with left SCN injury followed by left SON sectioning were lower than in left SCN injury or left SON sectioning groups treated; the animals with right SCN injury followed by right SON sectioning showed lower testosterone levels than the animals treated with right SON sectioning.

Estradiol levels were higher in rats treated with right SCN injury followed by right SON sectioning than in rats treated with right SCN injury or right SON sectioning or with left SCN injury followed by left SON sectioning (Figure 6).

The 2-way ANOVA test shows that animals treated with right SCN injury followed by right SON sectioning at 11.00 hours showed lower estradiol levels than rats treated at 17.00 hours ($F_{1, 23} = 12.91$; $P < .0015$) and with significant effects between treatments and hours ($F_{1, 23} = 20.74$; $P < .0001$).

Discussion

Present results indicate that on proestrus day the neural signals arising from the right or both SONs regulate on stimulating

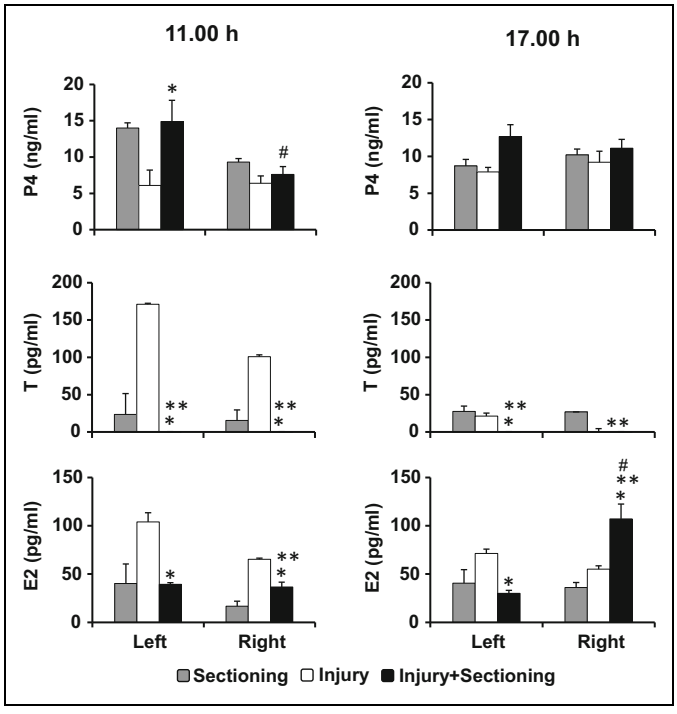

Figure 6. Effects on hormones levels resulting from the unilateral injury to the suprachiasmatic nucleus (SCN) followed by unilateral sectioning of the superior ovarian nerve (SON). Mean ± standard error of the mean of progesterone (P4, ng/mL), testosterone (T, pg/mL), and estradiol (E2, pg/mL) serum levels in rats treated with left or right SCN injury, left or right SON sectioning, or unilateral SCN injury followed by unilateral sectioning of the SON ipsilateral to the injured SCN (left or right injury + sectioning). Treatments were performed to rats on proestrus day between 11.00 and 11.30 or 17.00 and 17.30 hours. The animals were sacrificed 24 hours after treatment (n = 8 per group). *$P < .05$ versus corresponding SCN injured group; **$P < .05$ versus right sectioning group; #$P < .05$ versus left injury + sectioning (1-way analysis of variance followed by Tukey test).

way ovulation process, with a greater effect on the afternoon of proestrus. Hormone secretion is regulated differently on stimulating or inhibitory ways depending on the hormone studied and the time of day.

We have previously shown that in adult rats, the unilateral or bilateral sectioning of the SON resulted in lower ovulation rates and number of ova shed by ovulating animals.[33]

Injecting human chorionic gonadotropin (hCG) to adult rats with unilateral or bilateral SON sectioning treatment did not restore ovulation rates nor the number of ova shed by denervated ovaries.[33,34] In prepubertal rats with unilateral or bilateral SON sectioning, injecting hCG or pregnant mare serum gonadotropin (PMSG) or the sequential injection of PMSG followed 56 hours later with hCG did not restored ovulation.[35] The results of these studies suggest that the neural innervation provided by the SON modulates the reactivity of the follicular compartment to gonadotropins.

In the present study, the lower number of ova observed in rats treated with unilateral or bilateral SON sectioning at 11.00 or 17.00 hours suggests that for the normal response of preovulatory follicles to LH requires some types of neural information arriving through the SON. Since ovulation rates were not modified in rats treated at 11.00 but were lower in animals treated at 17.00 hours, we presume that the reduced follicular growth and lack of new corpora lutea are due to a lower sensitivity of the follicles to gonadotropins, and not to a failure in gonadotropins secretion.

According to Espey,[36] LH induces ovarian hyperemia, vasodilatation, edema, and even extravasation of blood in ovulatory follicles. Zackrisson et al[37] concluded that acute blood flow reduction during the ovulatory interval reduces ovulation rate in the rat. Electrical stimulation of the SON decreases ovarian blood flow.[10] The SON carries fibers containing NA, NPY, and VIP.[5] Both NA and NPY stimulate the vasoconstriction of ovarian arteries, suggesting that a decrease in the ovarian blood supply may be related to increases in NA and NPY.[38] The electroacupuncture at the level of the segment from which the ovarian sympathetic nerve emerge (T9–T10 and L3–L5) increases NPY concentrations in the follicular fluid.[39] According to Yao et al,[40] VIP induced a dose-related relaxation of NA-precontracted vessels, while NPY markedly reversed the relaxations induced by VIP. Then, we presume that the lower ovulation rates and number of ova shed observed in rats with right or bilateral SON sectioning treatment at 17.00 hours were caused by the lack of NA resulting from SON sectioning treatment and the rearrangement of neural factors that modify the sensibility of the follicles to gonadotropin required for ovulation to occur.

Consequently, the present results may extend our knowledge concerning the neural regulation of ovulatory functions after the LH peak (17.00 hours of proestrus). This study is the first to show that after the LH peak the neural information from SON is crucial for successful ovulation. Thus, it is possible that circadian signals may regulate these ovarian neuroendocrine changes necessaries for ovulation, perhaps through neural signals from SCN.

In the classical study by Everett and Sawyer,[41] the scientists proposed that in rats on proestrus day, between 14.00 and 16.00 hours, there is "a 24-hour periodicity in the 'LH-release apparatus' of female rats, disclosed by barbiturate sedation." In other days of the estrous cycle, the presence of such periodicity in the LH-release apparatus was described by Domínguez and Smith.[42] According to Terasawa et al,[22] rats on proestrus treated with bilateral SCN injury in the morning (9.00-10.00 hours) did not show changes in ovulation the next day, though ovulation was blocked when the treatment was performed between 12.00 and 14.00 hours.[23] In the present study, rats with bilateral SCN injury treatment at 11.00 hours did not ovulate, suggesting that between 11.00 and 14.00 hours the signals from both SCN are essential for GnRH secretion and ovulation. This fact is supported by the observed restoration of ovulation after injecting LH–RH to animals with bilateral SCN injury.

In rats on proestrus day, the unilateral SCN injury (left or right) treatment at 09.00 hours resulted in anovulation.[43] Such effect was not observed in the present study, suggesting that the neural signals from the SCN that participate in ovulation regulation vary along the time of day.

In the present study, the right ovary of rats with right SCN injury treatment released lower numbers of ova, suggesting that the neural signals from the right SCN participate in regulating ovulation in a stimulating and lateralized way. This regulatory information may be carried through the SON, since right SON sectioning to animals with right SCN injury treatment at 11.00 hours increased the inhibitory effect on ovulation rate and number of ova shed by the right ovary resulting from the right SCN injury treatment.

Experimental data have suggested that the brain controls ovarian functions through multisynaptic pathways and that the left ovary receives denser innervation from the brain stem and the hypothalamus than the right ovary.[44]

Zhang and Aguilar-Roblero[25] showed the presence of a clear rhythm in the mean firing frequency in in vitro neurons from both SCN sides of male rats. When Zhang and Aguilar-Roblero compared the data between each side of the SCN they observed that the electrical activity from the left SCN was basically unimodal while was bimodal of the right side of SCN. These results were interpreted as a suggestion of asymmetrical activity patterns from each side of the SCN, supporting the hypothesis of independent oscillators in each SCN, and that the observed asymmetry could reflect a functional lateralization of brain organization.

In the present study, the effects on ovulation resulting from the SCN injury do not parallel the changes observed on steroid levels. This suggests that although the endocrine signals that regulate ovulation and hormones secretion are the same, the regulatory neuroendocrine mechanisms are dissimilar.

During the morning of proestrus, activation of the hypothalamus–pituitary–adrenal axis results in increases in adrenocorticotropic hormone, corticosterone, and progesterone serum levels.[45] We have previously shown that in rats on proestrus day, the adrenals are the main source of progesterone levels.[9,46] Consequently, we presume that the lower progesterone levels observed in rats with left SCN injury treatment is due to lower adrenal progesterone secretion.

Since the left SCN injury treatment at 11.00 hours did not modify testosterone levels, the low level of testosterone observed in rats with the right SCN injury treatment suggests that the neural information arriving to the ovaries from the right SCN is necessary for regulating testosterone secretion.

Similarly, since unilateral SCN injury treatment did not modify estradiol levels, it appears that the neural information from either SCN is sufficient to maintain normal estradiol secretion levels.

Present findings indicate that, irrespective of the time of day, the unilateral or bilateral SON sectioning did not modify progesterone; the bilateral SON sectioning treatment at 17.00 hours yielded higher testosterone levels; and estradiol levels were modified according to time of treatment and the nerve sectioned. As was proposed by Flores et al,[9] the present findings suggest that changes in ovarian steroid hormone levels resulting from SON denervation reflect changes in ovarian sensitivity to LH and that the effects also depend on the time of day of treatments.

According to Gerendai et al,[24] Domínguez and Cruz-Morales,[47] and Cruz et al,[48] the SON and the vagus nerve are neural pathways connecting the central nervous system and the ovaries. In the present study, we explored if the SCN uses to SON as part of one of the neural pathway regulating ovarian steroid hormones secretion. We found that unilaterally SON sectioning modifies the neural signals from the SCN regulating progesterone, testosterone, and estradiol secretion. These results suggest that the SON is one of the neural pathways connecting the SCN and the ovaries.

Several studies have shown that the neural mechanisms regulating ovarian steroid hormones secretion vary according to the hormone studied.[31,49] In an ex vivo celiac ganglia-SON-ovary left system model on proestrus day, adding NA or acetylcholine to the ganglion compartment predominantly induced the ovarian release of androstenedione and estradiol and inhibited progesterone release.[50] According to Uchida and Kagitani[11] and Kagitani et al,[51] the electrical stimulation of the right SON affects different neural mechanisms regulating testosterone and estradiol secretion. The activation of alpha1-adrenoceptors decreased testosterone levels, whereas the activation of alpha2-adrenoceptors reduced estradiol secretion. According to the Uchida and Kagitani,[11] the lower secretion of estradiol resulting from SON stimulation is independent of the reduction in testosterone secretion.

Rosas et al[52] showed that the effects on progesterone, testosterone, and estradiol serum levels resulting from VIPergic stimulation depend on the time elapsed between treatment and autopsy and that these effects vary along the estrous cycle. Twenty four hours after VIPergic stimulation of the left ovary on proestrus day yielded higher testosterone and estradiol levels, while stimulating the right ovary yielded higher estradiol levels.[52]

According to Garraza et al,[53] overstimulating the ovaries obtained from rats on diestrus-1 or diestrus-2 day with NA, NPY, or VIP for 30 minutes modifies progesterone secretion in different ways, depending on the neurotransmitter used and the day of the cycle treatment was applied. Ovaries from rats on diestrus-1 day incubated with NA, VIP, or NPY yielded lower progesterone levels than controls, whereas ovaries from rats on diestrus-2 yielded higher progesterone levels. The overstimulation with NPY + NA to ovaries from rats on diestrus-1 day resulted in lower progesterone secretion than ovaries treated with NPY alone. In turn, NPY + NA treatment to ovaries obtained from rats on diestrus-2 day blocked the increase of progesterone secretion with NPY alone. Overstimulation with VIP + NA did not modify the effects of VIP alone.[53] Then, since the SON provides the ovaries with neural information using NA, VIP, and NPY, the effects of SON sectioning represent the lack of this interaction of neural information using NA, VIP, and NPY as neurotransmitter on steroidogenesis regulation.

Taken together, the present results support Kennaway's[12] and Sellix's[13] suggestions that the sympathetic innervation of the ovary may be influenced by the SCN and that the SCN modulates the timing of the ovarian clock via autonomic

nervous cues. The present results also suggest that on proestrus day, the SCN regulates steroid hormones secretion by different mechanisms and that these mechanisms vary according to the hormone studied and the time of day. The information from the right SCN carried by the SON plays a stimulatory role in the follicle's reactivity to LH that results in ovulation. Neural information from the left SCN does not appear to be essential for this process.

Authors' Note

DAR, RD, and LM-L planned the experiments. DAR, EV, and AIG performed experiments. DAR, EV, RD, CM, and LM-L devised the study, participated in the discussion of the results, and cowrote the manuscript. All authors read and approved the final manuscript. All experiments were carried out in strict accordance with the Mexican Law of Animal Treatment and Protection Guidelines and followed the Mexican Official Standard NOM-062-ZOO-1999 specifications.

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Declaration of Conflicting Interests

The author(s) declare no potential conflicts of interest with respect to the research, financial, authorship, and/or publication of this article.

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